

# Low-dose cyclophosphamide selectively expands resident anti-tumor T-cells allowing in situ control of colorectal cancer

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**Abbreviations Used:** *b.d.*, *bis die (twice-a-day)*; *CPM*, *cyclophosphamide*; *ELISpot*, *enzyme-linked immunospot*; *FACS*, *fluorescence-activated cell sorting*; *Fluorospot*, *fluorescent immunospot*; *Foxp3*, *Forkhead box p3*; *IFN- $\gamma$* , *Interferon- $\gamma$* ; *mCRC*, *metastatic colorectal cancer*; *MDSC*, *myeloid-derived suppressor cell*; *PBMC*, *peripheral blood mononuclear cell*; *PPD*, *purified protein derivative*; *SEM*, *standard error of the mean*; *SFC*, *spot-forming cell*; *TAA*, *tumor-associated antigen*; *Treg*, *regulatory T-cell*; *TD*, *treatment day*.

## **Translational Relevance**

Foxp3<sup>+</sup> regulatory T-cells (Treg) are enriched within colorectal cancer (CRC). Whilst some studies have identified a survival advantage in patients with increased Treg numbers within tumors, depletion of these cells both in vitro and in animal models results in enhanced anti-tumor T-cell responses and better tumor control. Oral low-dose CPM has previously been noted to effectively deplete Treg, but its effects have not been evaluated in the setting of a controlled, randomized trial. Here, we present a comprehensive profile of peripheral lymphocyte populations in metastatic CRC patients enrolled onto a randomized phase I/II trial of low-dose CPM. We demonstrate that CPM depletes Treg, B- and NK-cell populations, with coincidental increases in polyfunctional tumor-specific T cells. Patients identified as having an increased anti-tumor immune response exhibited significantly longer progression-free survival. Hence CPM has a clear impact on CRC patients, warranting further investigation in patients with earlier stage disease to prevent relapse following primary resection.

## ABSTRACT

**Purpose:** Anti-cancer T-cell responses can control tumors, but immune-suppressive mechanisms in vivo prevent their function. The role of Foxp3<sup>+</sup> regulatory T-cells (Treg) in metastatic colorectal cancer (mCRC) is unclear. We have previously shown depletion of Treg enhances CRC-specific effector T-cell responses. Low dose cyclophosphamide (CPM) targets Treg in animal models and some human studies, however the effect of CPM in mCRC was unknown.

**Experimental Design:** Fifty-five mCRC patients were enrolled onto a phase I/II trial and randomized to receive two week-long courses of low-dose (50mg b.d.) CPM or not. The absolute number, phenotype and anti-tumor function of peripheral blood-derived lymphocyte subsets were monitored throughout the course of treatment, along with 18-month follow-up.

**Results:** Initially CPM reduced proliferation in all lymphocyte subsets, however, a rapid mobilization of effector T-cells overcame this decrease, leading to increased absolute T-cell numbers. In contrast, a reduction in proportional and absolute Treg, B-cell and NK-cell numbers occurred. The expansion and subsequent activation of effector T-cells was focused on tumor-specific T-cells, producing both granzyme-B and IFN- $\gamma$ . CPM-treated patients demonstrating the most enhanced IFN- $\gamma$ <sup>+</sup> tumor-specific T-cell responses exhibited a significant delay in tumor progression (HR=0.29, 95% CI 0.12-0.69,  $P=0.0047$ ), compared to non-responders and no-treatment controls.

**Conclusions:** CPM-induced Treg-depletion is mirrored by a striking boost to anti-tumor immunity. This study provides the first direct evidence of the benefit of naturally primed T-cells in mCRC patients. Our results also support the concept that non-mutated self-antigens can act as useful targets for immunotherapies.

## INTRODUCTION

Despite recent advances, metastatic colorectal cancer (mCRC) remains the second leading cause of death from cancer in the United Kingdom (1) (1). The current care of patients revolves around excision of the tumor, histopathological tumor staging, and adjuvant 5-fluorouracil (or capecitabine) based treatment, or as palliative chemotherapy for patients with advanced metastatic disease. Chemotherapy has a significant morbidity (and even mortality) associated with its use, and ~40% of patients with “curative” treatment relapse and succumb to the cancer. Hence the drive to develop less toxic and more targeted therapies.

Correctly harnessing a patient’s immune system to target and kill cancerous cells has enormous potential but has thus far proven challenging, with immune-based therapies for CRC in particular lacking in efficacy (2). One potential obstacle to achieving objective anti-tumor responses is the suppression of tumor-specific T-cells by Foxp3<sup>+</sup> regulatory T-cells (Treg) (3,4). Cancer-bearing individuals have increased frequencies of Treg, both in peripheral blood, and enriched within the tumor microenvironment (5). We have previously shown that CRC can drive this expansion of Treg that control anti-tumor immune responses (6-10). Specifically, when peripheral blood-derived Foxp3<sup>+</sup> Treg from CRC patients are depleted in vitro, a resulting enhancement of tumor-antigen (5T4, CEA)-specific IFN- $\gamma$ <sup>+</sup> responses is found (11,12).

The utilization of metronomic low-dose cyclophosphamide (CPM) was initially proposed as a salvage therapy in end-stage cancer patients, aimed at inhibiting tumor angiogenesis (13-15), although the immunomodulatory effects of CPM had been noted decades earlier in animal models (16,17). Currently, there is a revived interest in using CPM for the purpose of depleting Treg in humans ((8,18) and reviewed in (19)). Foxp3<sup>+</sup> Treg are thought to be more susceptible to the toxic effects of CPM due to their low levels of intracellular ATP and glutathione, relative to other T-cells (20); glutathione is required to counteract the toxic effects of CPM on cellular DNA, thus giving effector Foxp3<sup>-</sup> T-cell

populations a survival advantage. Profound effects on other immune cell types, including NK cells (21), dendritic cells (22) and MDSCs (23) have also been noted. However, at least one group did not observe Treg depletion using a low-dose metronomic dosing regimen (24), and a lack of consensus exists due to the concurrent use of other immune-potentiating therapies; the heterogeneous methods used to identify Treg; the study days on which immunological responses were monitored; and the cohorts of cancer patients studied. In addition, many groups administer low doses of CPM intravenously, and differential pharmacodynamic effects with regards to Treg depletion (possibly due to the amount and bioavailability of CPM administered) are apparent, when compared to oral dosing (21,25).

Here, we sought to determine the effect of oral metronomic low-dose CPM on lymphocyte subsets in patients with metastatic colorectal malignancy, as part of a randomized phase I/II clinical trial. A key objective was to identify whether CPM treatment induced oncofetal antigen (5T4)-specific lymphocyte responses; the secondary objectives included measurements of the effects of CPM on T-cell subsets (i.e. number, phenotype and function) and tumor progression.

## **PATIENTS AND METHODS**

### **Patients**

55 subjects with inoperable mCRC were enrolled on to the phase I/II clinical trial, TaCTiCC (TroVax® and Cyclophosphamide Treatment in Colorectal Cancer; EudraCT: 2010-024380-41) (38). Patients had to show evidence of responding or stable disease within 4-weeks of trial entry, as demonstrated by CT scan in comparison to pre-treatment CT scan using RECIST criteria to measure quantitative tumor burden. Additional inclusion criteria included age  $\geq 18$  years, WHO performance status 0-2, lymphocyte count  $\geq 500/\mu\text{l}$ , neutrophil count  $>1200/\mu\text{l}$  and platelet count  $>100,000/\mu\text{l}$ . Key exclusion criteria included life expectancy  $<3$  months or patient relapse / evidence of active malignancy, bilirubin level  $>50\mu\text{mol/L}$ , completion of first-line chemotherapy less than 2 weeks from start of treatment, clinically apparent / active autoimmune disease or those receiving immunosuppressants. All patients gave informed consent personally.

### **Study Design & Treatment**

This trial was a randomized, open-label study carried out from September 2012 to May 2016. Randomization was undertaken at the Clinical Trials Office, University Hospital of Wales, using an un-stratified balanced block design, with the outcome communicated to the attending physician immediately upon randomization. Patient characteristics are shown in Table 1. Upon enrolment, 27 patients were randomized to a cyclophosphamide group, and 28 were randomized to a no treatment control group. One patient from the watch and wait group withdrew consent before receiving allocated intervention, and two patients were later found to have had a curative procedure pre-enrolment, thus 25 patients were analyzed from this group and reported in this manuscript, as shown in the CONSORT flow diagram (Supplementary Figure 6).

Orally administered 50mg CPM was taken twice a day on treatment days 1-7 and 15-21; no CPM was taken on treatment days 8-14 or 22-106, or until patient relapsed. Peripheral blood samples (10-40mls) were taken at regular intervals during therapy, as shown in the schematic (Figure 1A). Bloods were consequently taken at treatment days 29, 43, 64, 78 and 106. All patients were clinically examined throughout the trial, with progression-free survival monitored.

### **Lymphocyte Purification and Culture**

Peripheral blood samples were collected in 10ml lithium heparin tubes (BD Biosciences). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation of heparinized blood over Lymphoprep (Axis-Shield). Cells were then washed and re-suspended in advanced RPMI (Life Technologies) supplemented with 5% batch-tested, pooled human AB serum (Welsh Blood Service), L-glutamine and penicillin / streptomycin. PBMC were plated in 96-well plates (Nunc) and cultured in triplicate wells with specific antigens for 14 days, supplemented with 10 $\mu$ l CellKine media (Helvetica Healthcare) on day 3 and fresh media containing 20 IU/ml IL-2 on days 7 and 10.

### **Antigens**

Forty-one 20mer peptides overlapping by 10 amino acids covering the entire human 5T4 protein were synthesized by Fmoc chemistry to >95% purity (GL Biochem, Shanghai, China). The peptides were divided into 13 pools (Supplementary Figure 4). Whole 5T4 protein was produced as previously described (39). The recall antigen PPD (Purified Protein Derivative) (Statens Serum Institut, Denmark) and the T-cell mitogen PHA (Sigma) were used as positive controls. All antigens were used at a final concentration of 5  $\mu$ g/ml.

### **ELISpot Assays**

Polymer-backed 96-well filtration plates (MAIPS4510; Merck Millipore) were used for all ELISpot assays. Antibodies and alkaline phosphatase substrate kits were obtained from Mabtech. The concentrations of antibodies used and washing steps were according to the manufacturer's instructions; all antibody incubations were with 50µl / well. Cells were pooled from triplicate wells in identical culture conditions, washed, re-suspended and counted before being plated with or without the corresponding 5T4 peptide pool for direct comparison. The plate was incubated at 37°C, 5% CO<sub>2</sub> for 24 hours. Cytokine-producing T-cells were enumerated at the single-cell level by counting the number of spots per well using an automated ELISpot plate reader (ImmunoSpot® S6 Ultra; CTL Europe GmbH). Positive responses were identified as having at least 20 spot-forming cells (SFC) per 10<sup>5</sup> cultured cells, and at least double the number of spots above background.

### **FluoroSpot Assays**

PVDF 96-well filtration plates designed for low-autofluorescence (IPFL; Millipore) were used for all fluorospot assays. Antibodies to IFN-γ and granzyme B, and fluorescence enhancer kits were obtained from CTL Europe. All antibody incubations were with 100µl / well. Frozen PBMC were thawed and rested overnight in RPMI + 5% AB serum. Cells were then washed, plated, and stimulated with 5 µg/ml antigen in duplicate wells. Plates were incubated at 37°C, 5% CO<sub>2</sub> for 24 hours. Cytokine-producing T-cells were enumerated using Smart Count™ settings on an automated fluorospot plate reader (ImmunoSpot® S6 Ultra; CTL Europe GmbH), allowing for an assessment of single and dual-cytokine producing cells. Positive responses were identified as having at least 5 SFC/2x10<sup>5</sup> PBMC, and at least double that of the negative (no antigen) control.

### **Flow Cytometry**



A no-wash, single platform approach was utilized to perform T-cell and NK-cell counts. 3 $\mu$ l of CD3-APC (Miltenyi-Biotec), CD4-PE (BioLegend), CD8-PerCPCy5.5 (BioLegend), CD19-FITC (BioLegend) and CD56-Brilliant Violet-421 (BD) antibodies were added to a FACS tube, followed by 50 $\mu$ l of whole heparinized blood using a reverse pipetting technique. Samples were left at 4°C for 30 minutes, before addition of 450 $\mu$ l red blood cell lysis buffer (BioLegend). After 10 minutes, 50 $\mu$ l of CountBright™ Absolute Counting Beads (Life Technologies) were reverse pipetted into the lysed blood sample before acquisition on a FACSCanto II (BD). Forward scatter and side scatter threshold parameters were set on the instrument to exclude debris and a minimum of 5000 beads were collected. FACS samples were analyzed on FlowJo version 10 (TreeStar), and cell counts calculated based on number of gated events (i.e. cells) divided by number of gated beads in sample, multiplied by number of beads/ $\mu$ l in CountBright™ vial.

To analyze phenotypic markers expressed on freshly isolated peripheral blood mononuclear cell-subsets, two panels were devised; a regulatory T-cell panel consisted of CD4-APCh7 (BD), CD25-Brilliant Violet-421 (BioLegend), Foxp3-APC (eBioscience), Helios-FITC (BioLegend), Ki67-PE (BioLegend), HLA-DR-PECy7 (BioLegend) and ICOS-PerCPCy5.5 (BioLegend) antibodies. The second panel, to study cytotoxic lymphocytes, consisted of CD3-APCh7 (BD), CD56-Brilliant Violet-421 (BD), CD8-PerCPCy5.5 (BioLegend), Perforin-FITC (BioLegend), Granzyme B-Alexa Fluor 647 (BioLegend), HLA-DR-PECy7 (BioLegend) and Ki67-PE (BioLegend) antibodies. Live/Dead-Aqua (Life Technologies) was used in both panels to exclude dead cells. Surface staining was performed in phosphate buffered saline (PBS), 2.5% fetal calf serum (FCS) and 5mM EDTA; intracellular staining was performed in permeabilization buffer plus 2% normal rat serum. Samples were acquired on a FACSCanto II (BD) and data analyzed using FlowJo version 10 (TreeStar).

## **Statistics**

Follow-up is complete to Dec 13, 2016, by which point all patients had progressed or were receiving other treatments. All analyses were performed on an intention-to-treat basis with patients ineligible for the trial excluded. Datasets were tested for normality, and appropriate tests were used to compare patient immune responses at specified time-points during the trial (non-parametric: Wilcoxon signed rank test, parametric: paired T-test). Error bars represent mean  $\pm$  standard error of the mean. PFS was analyzed using log-rank tests and displayed using Kaplan–Meier plots. A P-value less than 0.05 was considered statistically significant and all tests of significance were 2-sided. Analyses were performed using SAS v9.4 and GraphPad Prism v7.

## **Study Approval**

Results reported in this manuscript are derived from a clinical trial approved by the Cardiff and Vale Ethics committee and the Gene Therapy Advisory Committee (GTAC 175). All patients gave full written, informed consent personally prior to inclusion on the trial.

## **Trial Registration**

ISRCTN registry number: ISRCTN54669986. UK Clinical Research Network Study number: 13014. European Union Drug Regulating Authorities Clinical Trial Number: 2010-024380-41.

## RESULTS

### Patient Baseline Characteristics

One patient from the no treatment group withdrew consent before receiving the allocated intervention, and two further patients from this group were later found to have undergone a curative procedure pre-enrolment. These three patients were not included in the analyses of immune responses and progression-free survival (PFS), however an additional patient was recruited to this group and was included in the analyses, hence 52 patients could be evaluated. All mCRC patients presented with stable metastatic disease in at least one location, primarily liver, lung and peritoneal metastases; further baseline characteristics of the patients randomized to each group are shown (Table 1).

### Effects of CPM on T-cell and NK cell activation and frequency

Over the 22-day course of low-dose cyclophosphamide treatment, 40ml blood samples were collected at days 1, 8, 15 and 22 for analysis of 5T4-specific T-cell and antibody responses, and 10ml blood samples collected at days 1, 4, 8, 15, 18 and 22 to analyze phenotype and function of lymphocytes. 40ml blood samples were taken from control patients at treatment days 1 and 22, as shown in the treatment overview (Figure 1A).

The regulatory T-cell proportion was identified by FACS, first gating on live lymphocytes, then drawing a quadrant to distinguish CD4<sup>+</sup>Foxp3<sup>+</sup> from CD4<sup>+</sup>Foxp3<sup>-</sup> T-cells, a representative example of which is shown (Figure 1B). There was a significant decrease in the proportion of CD4<sup>+</sup> T-cells expressing Foxp3 at treatment day 4 in comparison to baseline (TD1 mean 11.54 ±1.25% vs. TD4 10.24 ±1.03%,  $P=0.0002$ ), however this quickly recovered to baseline levels by treatment day 8 (Figures 1B-C). A smaller proportional reduction was also noted at treatment day 18, although this did not reach significance. Whilst the reduction on Treg proportion was transient, a more sustained decrease in absolute Treg number was

observed. Peripheral Treg numbers significantly decreased by treatment days 15 (TD1 mean  $60.54 \pm 5.58$  cells /  $\mu\text{L}$  vs. TD15  $52.15 \pm 5.21$ ,  $P=0.018$ ) and 18 (TD1 mean  $60.54 \pm 5.58$  cells /  $\mu\text{L}$  vs. TD18  $49.77 \pm 5.78$ ,  $P=0.0089$ ) before starting to recover towards pre-treatment numbers at day 22 (Figure 1D).

The proportion of Foxp3<sup>+</sup>CD4<sup>+</sup> Treg expressing Helios, a putative marker of thymically-derived Treg (26), was measured. A representative example of the gating, and reduced Helios proportion at TD4, is shown (Supplementary Figure 1A). The proportion of Helios<sup>+</sup> Treg dipped between TD1-4 (TD1 mean  $75.1 \pm 1.40\%$  vs. TD4  $73.71 \pm 1.35$ ,  $P=0.30$ ) and then dipped more significantly at TD15-18 (TD15  $77.68 \pm 1.41\%$  vs. TD18  $74.30 \pm 1.45$ ,  $P=0.0001$ ; Figure 1E). Each of these dips in Helios<sup>+</sup> T-cells were followed temporally 4 days later by a similar significant dipping in the proportions of proliferating Treg, as demonstrated by Ki67 staining (TD1  $17.27 \pm 1.46\%$  vs. TD8  $14.49 \pm 1.26\%$ ,  $P=0.0048$ ; TD15  $18.64 \pm 1.37\%$  vs. TD22  $15.96 \pm 1.17\%$ ,  $P=0.043$ ) (Figure 1F). These results corroborate a previous report implicating Helios as a marker of activated, proliferating Treg (27). Indeed, it seems clear that CPM is targeting these activated Treg, as both the proportional reduction and depletion of Treg correlates with the pre-treatment proportions of Helios<sup>+</sup> (data not shown) or Ki67<sup>+</sup> Treg (Figures 1G and 1H). These data demonstrate an enhanced susceptibility of actively proliferating Treg cells to the cytotoxic effects of CPM.

The relationship between the proportion (percentage) of Ki67<sup>+</sup> cells in blood and absolute cell numbers is more complex for CD3<sup>+</sup>CD4<sup>+</sup>(Foxp3<sup>-</sup>) T-cells, CD3<sup>+</sup>CD8<sup>+</sup> T-cells, NK-cells and B-cells. During the first cycle of CPM in week one, all populations except B-cells show an increase in blood cell numbers (Figure 2A-C and Supplementary Figure 2), this rise being most marked for CD3<sup>+</sup>CD8<sup>+</sup> T-cells (TD1 mean  $331.6 \pm 41.5$  cells/ $\mu\text{L}$  vs. TD4  $457.5 \pm 81.39$ ,  $P=0.0039$ ; Figure 2B); this corresponded with a fall in Ki67 expression in all lymphocyte populations (Figure 2D-F). This inverse relationship is different to that observed

within the Treg population and indicates mobilization into blood from a reservoir occurs early for this rapid increase in cell numbers. These numbers quickly returned to baseline, and this inverse effect was not repeated in the second cycle of CPM as the %Ki67 expression now increased for all three cell populations (in contrast to Treg where %Ki67 expression falls during second CPM cycle), although with little effect on overall cell numbers. Given that Treg numbers remained stable but CD4<sup>+</sup>Foxp3<sup>-</sup> T-cell numbers increase at TD4, this contributes to the proportional reduction in Treg as described above.

Whilst effector T-cell numbers remained relatively stable from treatment day 8 onwards, the numbers of peripheral CD3<sup>-</sup>CD56<sup>+</sup> NK-cells steadily decreased after the first week (TD1 mean 97.0±15.1 cells/μL vs. TD15 73.5±14.8, *P*=0.0036, vs. TD18 77.4±14.3, *P*=0.03, vs. TD22 63.9±11.9, *P*=0.0003; Figure 2C). Overall, these data mirror the time-points at which significant depletion of Treg numbers was also observed; hence, CPM does not appear to only selectively deplete Treg. A similar profile was noted amongst B cells, with significant depletions by TD18 (TD1 mean 134.5±37.8 cells/μL vs. TD18 82.1±23.1, *P*=0.036; Supplementary Figure 2).

As mentioned above, the proportion of potentially proliferative Ki67<sup>+</sup> T- and NK-cells all significantly decreased during the first CPM treatment week, but then reversed and dramatically increased from day 8 and continued to increase into the second cycle of CPM (TD15-22) (Figures 2D-F), with significant increases in %Ki67 seen in CD4<sup>+</sup>Foxp3<sup>-</sup> T-cells (TD1 mean 3.68 ±0.34% vs. TD18 4.28% ±0.43, *P*=0.045; Figure 2D); CD8<sup>+</sup> T-cells (TD1 mean 4.22 ±0.51% vs. TD22 5.92 ±0.63, *P*=0.0012; Figure 2E) and NK cells (TD1 mean 8.22 ±0.48% vs. TD22 12.70 ±1.58, *P*=0.001; Figure 2F). This effect was observed in 23/27 patients and is mirrored by the absolute reduction in Treg numbers at TD15-22 (Figure 1D), raising the possibility that Treg are controlling homeostatic proliferation / activation of these other cell populations. The striking increases in %Ki67 corresponded to T-cell activation

demonstrated by increased expression of HLA-DR, ICOS and CD25 (Supplementary Figure 3).

### **Increased lymphocyte cytotoxicity with CPM treatment**

CPM treatment leads to a marked expansion of CD8<sup>+</sup> T-cells. To explore the impact of this treatment on the function of these cytotoxic T-cells, we measured the cytolytic potential of peripheral CD8<sup>+</sup> T-cells by intracellular perforin and granzyme-B, both of which play a major role in cell-mediated cytotoxicity of cancer cells (28). Corresponding with increased number, proliferation and activation of CD8<sup>+</sup> T-cells, the intracellular expression of both molecules significantly increased during CPM treatment, an effect noted for the majority (24/27) of patients taking CPM (perforin TD1 mean 32.31 ±4.44% vs. TD8 38.54 ±4.40%, *P*=0.0071; Figure 3A, and granzyme-B TD1 mean 53.37±5.38% vs. TD8 56.83±5.32% , *P*=0.0084, vs. TD18 60.63±5.35%, *P*=0.0009; Figure 3B); representative examples of FACS staining are shown (Supplementary Figures 1B and 1C). A similar increase in cytotoxicity potential (i.e. expression of granzyme-B / perforin) was found in NK-cells, an effect of CPM not previously described (data not shown).

To assess whether the increased intracellular expression translated to an increase in functional cytotoxicity i.e. actual release of granzyme-B, PBMC from patients, identified as having an increased expression of granzyme-B at TD4, were stimulated ex vivo with a peptide library covering the tumor antigen 5T4, in a fluorospot assay. This dual color (analyte) assay determined the production of the T<sub>H</sub>1 cytokines IFN- $\gamma$  (represented as green spots) alongside granzyme B (represented as red spots), allowing for cells with multifunctionality to be enumerated. Representative fluorospot well images for two patients are shown (Figure 3C). The number of T-cells producing granzyme-B per 10<sup>5</sup> PBMC, either alone or in combination with IFN- $\gamma$ , increased markedly in both patients whilst taking CPM (Figure

3D). These data reveal effector anti-tumor T<sub>H</sub>1 function and cytolytic capability increases within a very short time frame after commencing CPM.

### **Augmented Anti-5T4 T-cell Responses During CPM Treatment Correlate with Prolonged Progression Free Survival**

T<sub>H</sub>1-type (IFN- $\gamma$ )-producing T-cells are thought to be important mediators of beneficial anti-tumor immune responses (12). The oncofetal antigen 5T4 has been proposed as a target antigen given its expression on the majority of gastrointestinal adenocarcinomas, and the ability of 5T4-specific T-cells to recognize and kill 5T4-expressing tumor cells (29). We have also found anti-5T4 CD4<sup>+</sup> T-cell responses are associated with control of CRC, and are in turn regulated by tumor-driven Treg (6,8). CPM led to specific increases in T-cell responses to 5T4 in CRC patients. Positive 5T4 peptide epitopes were identified using a peptide pool matrix system (Supplementary Figures 4A and 4B). Example ELISpot well images from three patients (143, 151 and 152) taking CPM demonstrate how a negative response at TD1 to 5T4 peptide pools became highly positive by TD8, indicative of a de novo or unmasked response to single 5T4 epitopes (Figure 4A). Significant increases in the overall magnitude of anti-5T4 T-cell responses were generated peaking at day 15 (TD1 331.9  $\pm$ 65.1 SFC/10<sup>5</sup> vs. TD15 630.9  $\pm$ 95.7,  $P=0.0013$ ; numbers reflect cognate T-cell responses as measured by the number of 5T4-specific IFN- $\gamma$  spot forming T-cells i.e. SFC/10<sup>5</sup> cultured PBMCs; Figure 4B). In patients followed up for a further 3 months after CPM treatment, who were taking no other medication (schematic shown; Supplementary Figure 5A), CPM-induced 5T4-specific T-cell responses remained at a high level until treatment day 43; following this, responses returned to their base-line levels throughout the rest of the trial (Supplementary Figure 5B). There was no evidence of immunocompromise in the patient groups (i.e. controls and CPM treated subjects) despite the advanced nature of the tumors. T-cells were responsive to control antigens such as tuberculin PPD (Figure 4C) and influenza haemagglutinin (data not shown).

Overall, 19 out of 27 patients responded to CPM with a mean increase (at TD8, 15 and 22) in total 5T4 T-cell responses above the lower 95% CI of 105 IFN- $\gamma$ <sup>+</sup> SFC/10<sup>5</sup> cultured PBMC (Figure 4D), and the magnitude of this response was so large that on an intention to treat basis, these increased responses remain highly significant even when including the non-responders in the analysis (Figure 4B). Consistent increased anti-5T4 T-cell responses significantly correlated with mean increases in CD3<sup>+</sup> T-cell numbers during the course of treatment, whereby those patients with a tendency for lymphodepletion during CPM treatment failed to mount robust anti-5T4 immune responses ( $P=0.011$ ; Figure 4E).

We next looked at how patients responding to CPM treatment with increased 5T4-specific immune responses fared clinically compared to subjects who did not respond to CPM or who were randomized to the control group. Disease progression was assessed by radiological progression of the tumor or metastases; increase in tumor markers; or clinical deterioration. Eight patients randomized to CPM failed to respond (black circles, Figure 4D and E). Progression free survival of both groups was assessed, based on the time taken from study enrolment to evidence of tumor progression, as assessed clinically, or from radiological progression (30); the Kaplan-Meier curves are shown (Figure 4F). A significant difference was found between CPM responders and non-responders plus eight control mCRC patients not taking CPM or any other treatment (HR=0.29, 95% CI 0.12-0.69,  $P=0.0047$ ; Figure 4F). Median time-to-progression of patients responding to CPM in this manner was 3.7 months versus 2.4 months for non-responders / controls.

In summary, the most striking effects of CPM therapy given to individuals with inoperable mCRC, in terms of prolonging PFS, are seen when anti-tumor T-cell immune responses are induced via depletion of circulating Treg.



## DISCUSSION

The clinical effectiveness of cancer immunotherapy is reliant upon a number of factors that allow an immune response to overcome the tumor. A major reason for failure in spite of the presence of anti-tumor responses is the existence of tumor-driven immunosuppressive mechanisms, mediated, at least in part, by Foxp3<sup>+</sup> Treg. Cyclophosphamide has been used in pre-clinical and clinical studies(19), with it being examined for its immunological effects at both high and low doses. Whilst metronomic low-doses for cancer immunotherapy have been infrequently used, there are reports demonstrating prolonged Treg reduction (21,31). One non-randomized, retrospective study identified a possible small survival benefit in metastatic cancer patients when using low-dose CPM (32). However, these studies highlighted the need for in-depth characterization of CPM on immunological findings, to optimize anti-tumor immunity in potential combination with other treatments.

Following initial CPM administration to mCRC patients, we demonstrated a short duration in the reduction in Treg proportion and overall Treg number. CPM acts very rapidly, within days (potentially hours): the first measurement on day 4 of treatment revealed CPM had induced numerous immunological perturbations, including significantly increased T-cell numbers resulting in fluctuations of CD4:CD8 T-cell ratio; enhanced CD8<sup>+</sup> T-cell cytolytic function; and decreased expression of activation markers in Foxp3<sup>+</sup> Treg. Despite such pronounced immunological changes observed, nearly all patients taking CPM did not report any side effects during treatment (one patient experienced nausea). Whilst our understanding of the pharmacodynamics of CPM with regards to its effect on lymphocytes remains incomplete, it appears that CPM targets lymphocytes with greatest proliferative potential first, given the quite drastic reductions in Helios<sup>+</sup>Ki67<sup>+</sup> Foxp3<sup>+</sup> Treg seen almost unanimously amongst the trial participants. Indeed, patients with a higher pre-treatment expression of Helios (described as a marker of Treg activation and proliferation (27)) or %Ki67-expressing

Treg, exhibited a greater degree of proportional Treg reduction and absolute Treg depletion during CPM treatment. At the same time-points (TD15-18), significant depletions of CD56<sup>+</sup> NK-cells and B-cells were also observed. It should be noted that amongst lymphocytes, NK-cells, B-cells and Treg are highly dynamic proliferative populations *in vivo*, and homeostatic turnover is more rapid than for CD4 and CD8 effector T-cell populations (33-35). Thus, lymphocyte subsets with a relatively high proliferative capacity were more readily targeted by CPM. In this case however, reductions in absolute numbers of NK-cells or B-cells in the periphery did not appear to influence patient outcome, and NK-cell phenotype and cytolytic potential was actually augmented by CPM (data not shown).

Evidence for actual killing of Foxp3<sup>+</sup> Treg remains elusive; however from our experiments and previous reports (31), we hypothesize that CPM blocks activation and proliferation of Treg, thus resulting in the increased effector T-cell numbers seen *in vivo* and enhanced cytolytic T-cell functions *ex vivo* during treatment. In support of this, there is evidence indicating that reduced activation of Foxp3<sup>+</sup> Treg, as shown here during CPM treatment, leads to heightened immune responses via localized Treg : effector T-cell ratio imbalances (36). However, many of the effects seen on Treg were transient and the Treg proportion rebounds during treatment. This could be an effort to maintain peripheral homeostasis in the face of reduced Treg functionality. It could also be the result of T-cells initially being released at a greater rate from lymphoid organs (at TD1-4), before trafficking out from the periphery (at TD5-8) and into secondary lymphoid tissues or, potentially, tumors, as demonstrated when CPM is administered in mouse tumor models (22). Absolute Treg number only significantly reduced, i.e. depleted, at treatment days 15 and 18 before recovering, revealing the biphasic nature of Treg responsiveness to CPM and highlighting the importance of multiple immunological measurements throughout the treatment course.

The boosting of anti-tumor immunity by CPM is known to extend beyond Treg depletion; in a mouse model of colon carcinoma, not only were significant effects on dendritic cell homeostasis also noted, but enhanced anti-tumor responses mediated by CD8<sup>+</sup> T-cells were found after CPM administration, despite only transient Treg depletions (22). This is in stark contrast to a murine melanoma model, whereby perforin is down-regulated in CD8<sup>+</sup> TILs in mice receiving 2.5mg CPM (23). Here, we identified that the cytolytic potential and function of CD8<sup>+</sup> T-cells increased markedly during CPM treatment in the majority of patients. Indeed, utilizing a dual-color fluorospot assay, we were able to measure increased numbers of granzyme-B-producing 5T4-specific T-cells just 4 days into CPM treatment. These augmented responses result from enhanced functionality of T-cells, since single- and dual-IFN- $\gamma$  and granzyme B-producing T-cell responses also increased in response to the T-cell mitogen PHA (data not shown). However, given there was no increase in T-cell responses to the control antigens, in contrast to the striking increase in responses directed against 5T4, this is indicative of immunosuppression specifically targeting anti-tumor immune responses.

Although many of the immunological readouts returned to baseline by treatment day 8, crucially the anti-tumor immune response mediated by IFN- $\gamma$ -producing 5T4-specific T-cells was significantly enhanced over a more prolonged time period for the majority of patients, again indicative of reduced functional capacity of Treg and augmented effector T-cell responses. The production of IFN- $\gamma$  by the host plays a crucial role in the ultimate success of therapy, and has a direct role in inducing permanent senescence of tumor cells (37). Here, the critical readout of whether such responses are beneficial is seen in prolonged progression free survival of the patients, whereby those patients mounting increased anti-5T4 IFN- $\gamma$ <sup>+</sup> T-cell responses over the course of treatment had a significantly better clinical response in comparison to patients not responding to CPM or those receiving no treatment. This data demonstrates the power of a targeted, T<sub>H</sub>1-based immune response to an antigen

expressed on tumor cells, given that IFN- $\gamma$  responses to the irrelevant recall antigens had no effect on survival.

Low-dose CPM appears to be a safe and beneficial drug for end-stage mCRC patients with disseminated disease. Our on-going studies aim to evaluate its usefulness in boosting the immunotherapeutic potential of anti-cancer vaccination with a 5T4-containing pox-virus.

## **Author Contributors**

MS, RiH, RoH, AwG and AnG designed the study. MS, TP, DR, RA, AIB, RJ, SG and AnG did the study procedures. MS, AnB, DR, AT, KS and HB performed IFN- $\gamma$  ELISpot and flow cytometry. DB and RiH performed 5T4 ELISAs. MS, TP, AnB, DR, AT, DB and RiH collected data. MS and RoH managed data. MS, TP, RiH, RoH, AwG and AnG interpreted data. RoH did statistical analysis. MS, RoH and AnG wrote the report. MS, TP, AnB, DR, AT, KS, HB, RA, AIB, RJ, SG, DB, RiH, RoH, AwG and AnG reviewed the report.

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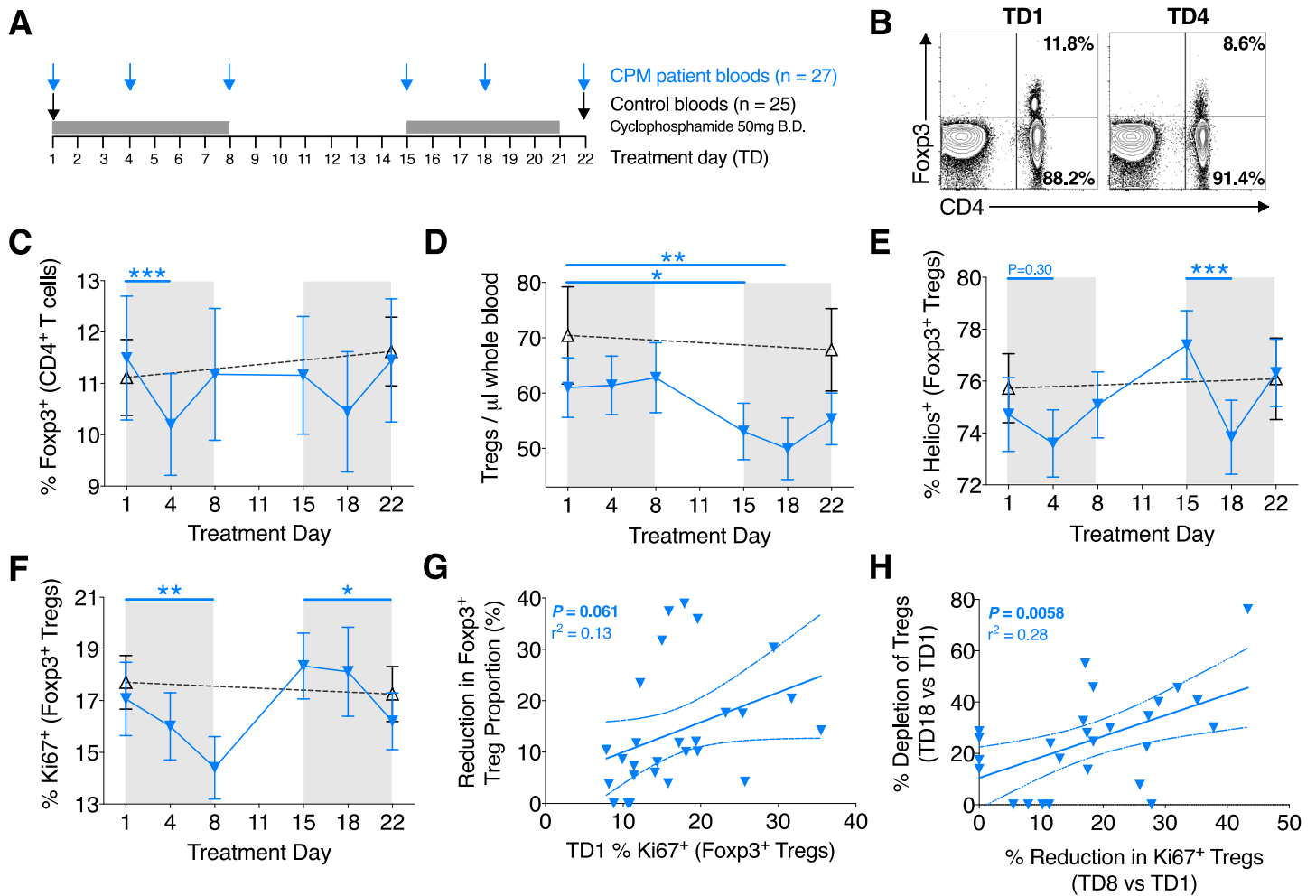
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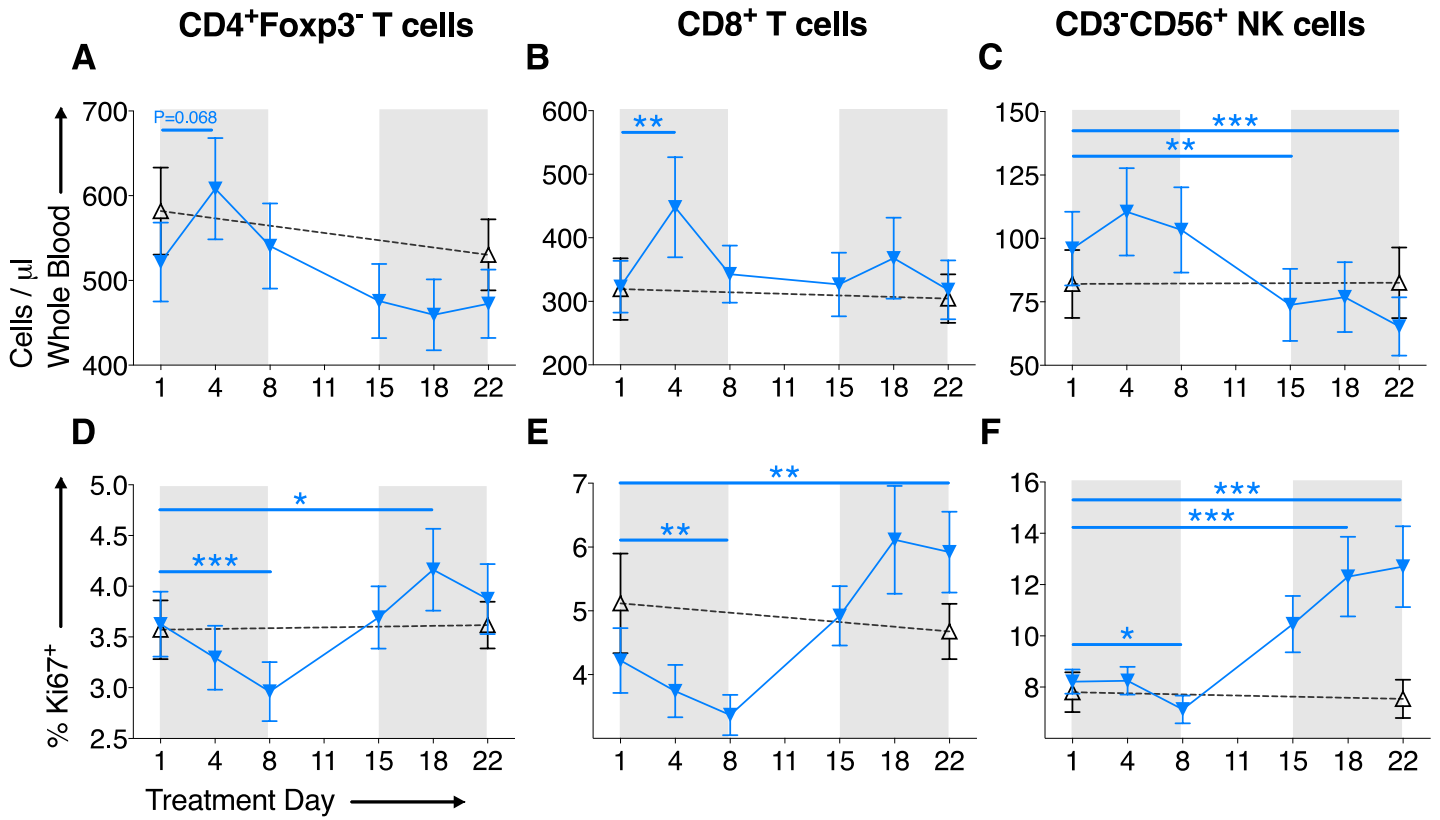
**Table 1. Patient baseline characteristics.**

	<b>CPM (n=27)</b>	<b>No CPM (n=25)</b>
Sex		
Female	9 (33%)	5 (20%)
Male	18 (67%)	20 (80%)
Mean age (years)	65.2	63.1
Site of Primary		
Right	5 (19%)	4 (16%)
Left	9 (33%)	12 (48%)
Sigmoid / Rectum	10 (37%)	9 (36%)
Not identified	3 (11%)	0 (0%)
Site of Metastases		
Liver	19 (70%)	14 (56%)
Lung	11 (41%)	13 (52%)
Peritoneum	11 (41%)	8 (32%)
WHO performance Status		
0	15 (56%)	10 (40%)
1	12 (44%)	14 (56%)
2	0 (0%)	1 (4%)
Previous treatment		
Primary colectomy	15 (56%)	17 (68%)
Capecitabine	27 (100%)	25 (100%)
5-Fluorouracil	24 (89%)	24 (96%)
Irinotecan	11 (41%)	11 (44%)
Oxaliplatin	9 (33%)	9 (36%)
Cetuximab	3 (11%)	0 (0%)
Aflibercept	2 (7%)	1 (4%)

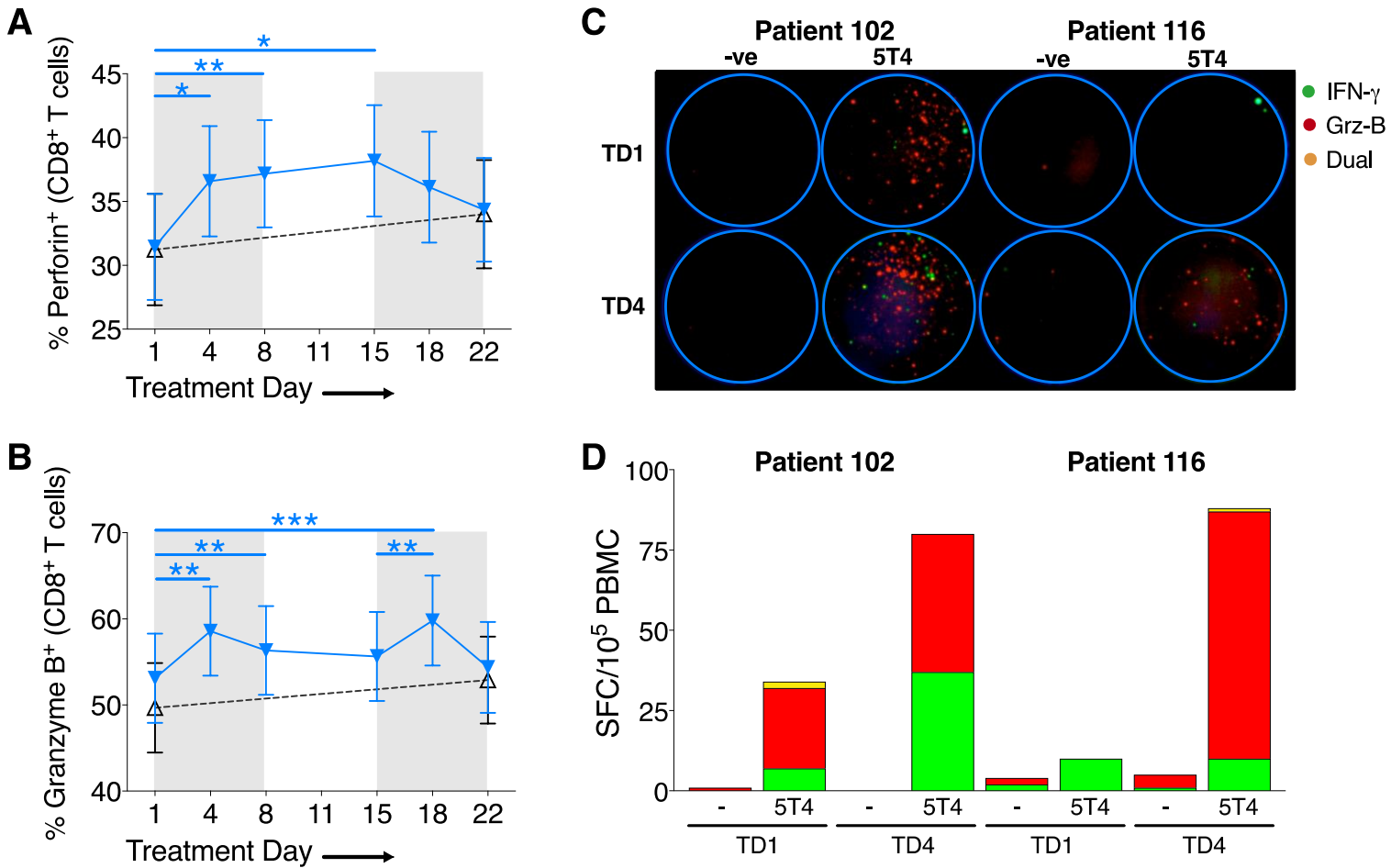


**Figure 1. The effect of metronomic, low-dose cyclophosphamide on Foxp3<sup>+</sup> regulatory T-cells.** (A) 50mg cyclophosphamide (CPM) was administered twice daily to 27 CRC patients on treatment days 1-7 and 15-21, with bloods being taken at regular intervals, as shown in this schematic. (B) Representative FACS plots of CD4 and Foxp3 expressing lymphocytes, and the gating used to denote Foxp3 percentage. (C) The percentage of Foxp3 expressing live CD4<sup>+</sup> T-cells was measured at the indicated time points throughout treatment. (D) Overall peripheral Treg numbers were derived from the number of CD3<sup>+</sup>CD4<sup>+</sup> T-cells from whole blood samples, and the proportion of CD4<sup>+</sup> T-cells expressing Foxp3 in subsequent phenotypic analysis. The percentage of live CD4<sup>+</sup>Foxp3<sup>+</sup> Treg expressing Helios (E) and Ki67 (F) was measured at the indicated time points throughout treatment. (G) The percent reduction in the proportion of Foxp3<sup>+</sup> Treg at treatment day 4 in comparison to baseline was correlated with the percent of CD4<sup>+</sup>FoxP3<sup>+</sup> Treg expressing Ki67 pre-treatment, i.e. treatment day 1. (H) The percent of overall depletion of Treg numbers at treatment day 18 in comparison to treatment day 1 was correlated with the percent reduction in Ki67-expressing Foxp3<sup>+</sup> Treg at treatment day 8 in comparison to treatment day 1. Blue lines indicate patients

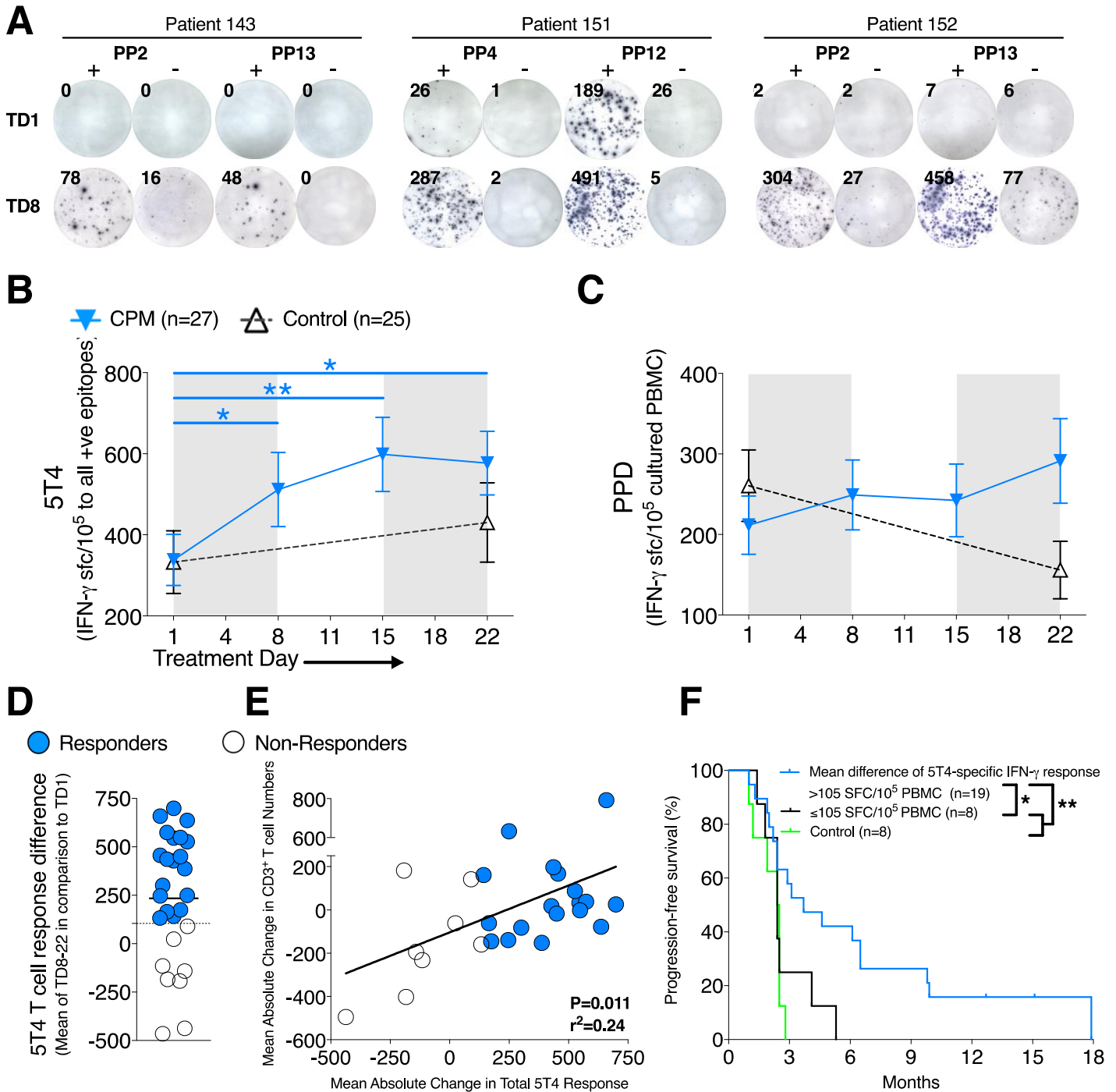
taking CPM (n=27); black lines indicate control patients at the same stage of tumor progression (n=25). Significant differences are indicated (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001).



**Figure 2. T- and NK-cell number and proliferation in response to cyclophosphamide.** The overall number of CD3<sup>+</sup>CD4<sup>+</sup>(Foxp3<sup>-</sup>) T-cells (A), CD3<sup>+</sup>CD8<sup>+</sup> T-cells (B) and CD3<sup>-</sup>CD56<sup>+</sup> NK cells (C) per μl whole blood were analyzed at indicated time points. The expression of Ki67 in CD4<sup>+</sup>(Foxp3<sup>-</sup>) T-cells (D), CD8<sup>+</sup> T-cells (E) and CD3<sup>-</sup>CD56<sup>+</sup> NK cells (F) was monitored throughout the course of CPM treatment. Blue lines indicate patients taking CPM (n=27); black lines indicate control patients at the same stage of tumor progression (n=25). Significant differences are indicated (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001).



**Figure 3. Enhanced CD8<sup>+</sup> T-cell function during CPM treatment.** The expression of the cytolytic molecules perforin (A) and granzyme B (B) in CD8<sup>+</sup> T-cells was monitored throughout the course of CPM treatment. Blue lines indicate patients taking CPM (n=27); black lines indicate control patients at the same stage of tumor progression (n=25). Significant differences are indicated (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001). A dual color fluorospot analyzing for the production of IFN- $\gamma$  (green spots), and granzyme B (red spots) was performed, and representative well images are shown from two patients pre-treatment (TD1) and during CPM treatment (TD4), with PBMC stimulated with 5T4 peptide pools (Patient 102: C; Patient 116: E). The average number from duplicated wells of single- and dual-cytokine producing T-cells in response to indicated stimulus, and normalized to SFC/10<sup>5</sup> PBMC, is shown (Patient 102: D; Patient 116: F).



**Figure 4. Augmented anti-tumor (5T4)-specific responses during CPM treatment associate with prolonged survival.** PBMC were cultured with 5T4 peptide pools spanning the entire protein (see Supplementary Figure 4 for peptide sequences and peptide pool matrix) and IFN- $\gamma$ <sup>+</sup> T-cell responses were measured by ELISpot. Representative examples from three patients at TD1 and TD22 are shown (A). The total number of spots to all positive identified 5T4 epitopes (B) and tuberculin PPD (C) were enumerated and normalized to

SFC/10<sup>5</sup> cultured PBMC. Blue lines indicate patients taking CPM (n=27); black lines indicate control patients at the same stage of tumor progression (n=25). Significant differences are indicated (\* P<0.05, \*\* P<0.01). (D) The mean change in 5T4-specific T-cell response at TD8-22 in comparison to TD1 was measured for all patients, with patients being separated into responders (blue, n=19) and non-responders (black, n=8) below the 95% CI of responses at +105 SFC/10<sup>5</sup> cultured PBMC. (E) This change in 5T4 T-cell response was correlated with the patient's mean change in absolute peripheral CD3<sup>+</sup> T-cell numbers during CPM treatment. (F) High anti-5T4 T-cell responses were associated with progression-free survival data. In addition, control patients who did not take any medication during the trial were also included for analysis (green lines; n=8). Significant differences are indicated (\* P<0.05, \*\* P<0.01).